Population genetic diversity of *Fasciola* spp. from Mpumalanga and KwaZulu-Natal provinces of South Africa

By

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville campus, South Africa.

Contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Signed: Prof. S. Mukaratirwa (Supervisor) Signed: Dr. O.T. Zishiri (Co-supervisor)

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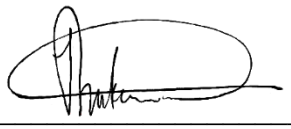

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ABSTRACT

Fasciola hepatica and *Fasciola gigantica* have been identified as the species causing fascioliasis in ruminants and humans with intermediate species being reported as well. Studies have shown an economic loss in excess of three billion United States dollars annually in the livestock sector due to infection by fasciolids. With the increase in importance of the disease, taxonomic classification and genetic characterization of *Fasciola* spp. is essential. Molecular markers have shown utility in both identification of species and elucidating phylogenetic patterns. Recent studies have shown utility of mitochondrial markers in elucidating genetic relationships and diversity due to their high variability and rapid analysis.

The current study was aimed at elucidating the evolutionary relationships and genetic diversity of *Fasciola* isolates from the KwaZulu-Natal (KZN) and Mpumalanga provinces in South Africa through analysis of the CO1 mitochondrial sequences. Fifty-five flukes were collected from abattoirs in the KZN and Mpumalanga provinces and DNA was extracted using the Phenol/Chloroform method. PCR amplification using the CO1 primers was performed with amplicons being sequenced at the Central Analytical Facilities of Stellenbosch University, South Africa. Resulting sequences were subjected to phylogenetic and diversity analysis. The study sequences were comparatively analysed with Genbank sequences from South Africa, Zimbabwe, Niger, Egypt and China; with Schistosoma japonicum as an outgroup.

Phylogenetic analysis showed that *F. hepatica* was present in all localities studied whilst *F. gigantica* was identified only in the Mpumalanga province. A 100% prevalence of *F. hepatica* was observed in KwaZulu-Natal and the high-veld region of Mpumalanga (21 and 17 isolates respectively). Thirteen (76%) of the seventeen flukes collected from the Belfast region of Mpumalanga were identified as *F. hepatica* while four isolates were identified as *F. gigantica*. A total of twenty-two haplotypes were identified with eighteen novel haplotypes being unique to the isolates from South Africa. Two novel *F. gigantica* haplotypes were identified with none of the study isolates sharing haplotypes with the Genbank isolates from China, Niger and Zimbabwe. Sixteen novel *F. hepatica* haplotypes were identified and one haplotype was shared between the experimental flukes and the Genbank isolates from China and Niger. Within the study samples, a number of haplotypes were restricted to a few individuals with a haplotype diversity of 0.89 indicating high diversity. Results from this study adds new knowledge to the genetic diversity of *Fasciola* species and its distribution in the KwaZulu-Natal and Mpumalanga provinces of South Africa.

Key words: Fasciola, Molecular markers, Phylogeny, Genetic diversity, Distribution, Lymnaea, Intermediate host, Geoclimatic factors

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CHAPTER 1: INTRODUCTION

1.1 Rationale for the research

Fascioliasis is a parasitic disease of high prevalence, affecting various mammals across the world (Mas-Coma *et al.,* 2003). This include but is not limited to domestic and wild ruminants as well as humans. The causative agents of this disease are two trematode species of the family Fasciolidae, *F. hepatica* and *F. gigantica* as reported by WHO (1995). *F hepatica* being of primary concern due to its nearly worldwide distribution, whereas the distribution of *F. gigantica* has been shown to be limited to Afro-Eurasia (Mas-Coma and Bargues, 1997). Fasciolids as well as their vector species possess immense capabilities in terms of colonization and as a result of this, the disease is able to spread rapidly (Mas-Coma *et al.,* 2003). Presence of *F. hepatica* has been reported in Africa, America, Asia, Europe and tropical islands of the Pacific (Mas-Coma *et al.,* 2009). *Fasciola gigantica* on the other hand, does not appear of be as efficient in colonization as evidenced from its geographical distribution which shows its presence in Africa and Asia and some parts of southern Europe (Mas-Coma and Bargues, 1997). Although Price (1953) noted the presence of *F. gigantica* in North America, it has not been cited in published literature over the last few decades. Prior to the 90's, human fascioliasis had been regarded as a zoonotic disease of secondary concern, however there has been an increase in reports all over the world. Reports have also shown that the prevalence, intensity of infection and geographical expansion has increased (Mas-Coma, 2004).

Fasciola hepatica and *F. gigantica* have been shown to similar life cycles (Mas-Coma and Bargues, 1997; Graczyk and Fried, 1999). The di-heteroxenous life cycle of *Fasciola* (Andrews, 1999) is dependent on Lymnaeid freshwater snails which have been shown to be the intermediate hosts of both *F. hepatica* and *F. gigantica* (Bargues *et al.,* 2001). Lymnaeids act as intermediate hosts to numerous other trematode species depending on the geographical region, ecology and snail-parasite specificity (Adam and Lewis, 1993; Esch and Fernandez, 1993; Toledo *et al.*, 1999).

1.2 Justification

Despite the highlighted importance of understanding phylogenetic patterns as well as population genetic structure of parasites and their intermediate hosts, limited knowledge has

been published in literature. With the increase in reports of fascioliasis and the rapid emergence of resistance to anthelmintics, further genetic studies are essential. Resistance to triclabendazole, the recommended treatment, has been reported from several parts of Eurasia (Brennan *et al.,* 2007; Fairweather, 2009; Peng *et al.,* 2009; Vilas *et al.,* 2012). Treatment and control of fascioliasis is largely dependent on early diagnosis of infection. Diagnosis of *Fasciola* infection in domestic ruminants has mainly focused on techniques using isolation of eggs from faecal samples with the fasciolid species being differentiated based on morphological characteristics of the flukes observed (Mas-Coma *et al.,* 2005; Thanh, 2012). Although widely used, corprological analysis shows low sensitivity in differentiation of *Fasciola* species especially using the eggs (Adedokun *et al.,* 2008). The increase in the occurrence of human and animal fascioliasis bring about an increase in the required sensitivity of diagnostic tools. The development and advancement of molecular genetic techniques and technology over the last decade has provided an efficient and cost effective identification of animal species.

Fasciola gigantica and *F. hepatica* have been shown to have an overlap in their geographical distribution (Mucheka *et al.,* 2015; Robles-Pérez *et al.,* 2015). This has resulted in numerous discussion and notions on the taxonomic patterns and identity of *Fasciola* spp. which resemble *F. gigantica* or *F. hepatica*, as well as observed intermediate forms (Periago *et al.,* 2007). The intermediate *Fasciola* form has been associated with several anomalies which include but are not limited to parthenogenesis, abnormal gametogenesis, variable ploidy and development of hybrids of different genotypes (Mas-Coma and Bargues, 1997). Several studies have confirmed cases of *Fasciola* hybrids using ribosomal and mitochondrial DNA sequence analysis (Itagaki and Tsutsumi, 1998; Itagaki *et al..*, 1998) in the far east, and recently in Africa. The overlapping distribution of *Fasciola* can be seen in some African countries (Ai *et al.,* 2011; Mucheka *et al.,* 2015). Studies have shown a concerning re-emergence of human fascioliasis in regions around the Nile Delta (Curtale *et al.,* 2000; Robinsons and Dalton, 2009).

The life cycle of *Fasciola* includes an intermediate host snail and a definitive host which is a mammal with complex reproductive biology and this results in high gene flow and genetic variability within *Fasciola* populations (Cwiklinski *et al.,* 2015). Studies show that multiple Lymnaeid snail species may be susceptible to *Fasciola* (de Kock *et al.,* 1989; Mas-Coma *et*

al., 2009) and this may also contribute to potential population sub-structuring as well as development of distinct genetic clusters between different geographical localities. How population structure may affect the spread of triclabendazole (TCBZ) resistance throughout a population has yet to be investigated (Cwiklinski *et al.,* 2015).

In order to fully elucidate and comprehend the impact and spread of any genetic change or mutation, which may be advantageous for survival in an organism, the genetic structure of the population must come under scrutiny. In the case of *Fasciola*, the population structure of *Fasciola* populations needs to be investigated using a large panel of polymorphic markers that exhibit neutral inheritance (Cwiklinski *et al.,* 2015). The population genetic information can be used to assist in studies on effects of population dynamics on host-parasite interactions and can be essential in development and modification of parasite control efforts (Cwiklinski *et al.,* 2015).

1.3 Aim

The aim of this study was to determine the population genetic structure of *Fasciola* spp. isolates from South Africa using molecular techniques.

1.4 Objective

The objective of this study was to describe the evolutionary relationships and population genetic diversity of *Fasciola* spp. collected from abattoirs in KwaZulu-Natal and Mpumalanga provinces of South Africa using cytochrome c oxidase sub-unit 1 (CO1).

1.5 Hypothesis

There is no genetic diversity among *Fasciola* spp. populations which overlap in geographical distribution.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Liver flukes of the genus *Fasciola*, have been identified as the causative agent of fasciolosis in both ruminants and humans (Mas-Coma *et al.,* 2003). Although a number of species under the genus *Fasciola* have been described, only *F. hepatica*, *F. gigantica* and *F. jacksoni* are recognized as taxonomically valid (Ai *et al.,* 2011). Whilst *F. hepatica* is mainly found in temperate areas and *F. gigantica* in tropical areas, however, an overlap of the two species has been reported in subtropical areas (Mas-Coma *et al.,* 2009). *Fasciola jacksoni*, commonly referred to as the fasciolid of Asian elephants, is less common and its phylogenetic position is yet to be elucidated (Caple *et al.,* 1978; Ai *et al.,* 2011). *Fasciola hepatica* and *F. gigantica* will be focused on in this review due to their wide distribution and increased importance in southern Africa as well as in other developing countries. This review focuses on phylogeny, genetic structure and the processes or factors which may influence the population genetic parameters of *Fasciola* species within the scope of this study.

2.2 Epidemiology of fascioliasis

Fascioliasis is a food and water-borne zoonotic disease with the causative agent being parasites from the genus *Fasciola* (Platyhelminthes: Digenea: Fasciolidae) (Mas-Coma *et al.,* 2003). Trematodes, by definition, are parasitic flatworms which possess unique life cycles which are divided into a sexual reproduction phase in their definitive host which may be mammals or other vertebrates, as well as an asexual reproduction phase in snail intermediate hosts (Keiser and Utzinger, 2007). These helminths can be divided into four groups with the grouping of the trematodes being dependent upon the final habitat of the parasite in humans. The four groups or categories are as follows: (a) the hermaphroditic liver flukes. These trematodes are mostly found in the bile ducts and infect the target host (ruminant or humans) upon ingestion of watercress, as seen with *Fasciola*, or raw fish in the case of *Clonorchis* and *Opisthorchis*; (b) the intestinal fluke (*Fasciolopsis*) which is hermaphroditic with infection occurring upon ingestion of water chestnuts contaminated with metacercariae by the definitive host; (c) the hermaphroditic lung fluke (*Paragonimus*) which can infect humans through ingestion of raw crabs or crayfish which harbor the metacercariae; and (d) the bisexual blood flukes (*Schistosoma*). These flukes inhabit the intestinal or vesical (urinary

bladder) venules with infection entering the host directly through the skin (Doughty, 1996; Keiser and Utzinger, 2009; Sithiathaworn *et al.*, 2009).

The intensity of infection and the degree of exposure to infective larvae share a direct proportionality due to the fact that the flukes do not actually multiply within the definitive host (Doughty, 1996; Mas-Coma and Bargues, 1997). In most cases, individuals that have been infected by flukes in endemic areas experience light to moderate infection intensity as shown by worm burdens. There are however, cases where a heavy worm burden can be observed in relatively small proportion of the population (Doughty, 1996). The infection intensity can also be linked to genetic predisposition with pathology being resultant of the worms themselves or the secreted eggs (Doughty, 1996).

A shift in the epidemiology of *Fasciola* has been observed over the last few decades (Mas-Coma *et al.*, 2009). In some areas, there has been a significant decline in the prevalence of infection by these trematodes (Keiser and Utzinger, 2009; Ezatpour *et al.*, 2015). This has been largely attributed to factors such as urbanization, social and economic development, implementation of health education programs, increased and more efficient food inspections, use of chemical fertilizers in agriculture, and water pollution trends (Keiser and Utzinger, 2009; Broglia and Kapel, 2011). In several other areas, however, an increase in frequency of infection has been observed (Mas-Coma *et al.*, 2009). Some of the major driving forces behind this have been the expansion of aquaculture to accommodate fresh water fish and crustacean production as a food source; as well as the advances in transportation and distribution systems used in expanding trade into local and international markets (Keiser and Utzinger, 2005).

2.3 Life cycle of *Fasciola* spp.

Fasciola hepatica and *F. gigantica* have a similar life cycle (Graczyk and Fried, 1999) (Figure 1). These hermaphroditic liver flukes infect mainly ruminants but may also incidentally, infect humans (Mas-Coma *et al.*, 2009). The life cycle of both helminths occurs in four distinct phases. These phases of the life cycle have been shown to be influenced by environmental factors as well as human activity (Mas-Coma and Bargues, 1997; Mas-Coma *et al.*, 2003). The phases of the life cycle occur as follows:

I. In the initial phase, the definitive host becomes infected through ingestion of the metacercariae encysted on water plants such as wild watercress (Hotez *et al.*, 2012). Within an hour of ingestion, the metacercariae undergo excystation inside the small intestine and then penetrate the host intestinal wall. They then migrate to the abdominal cavity within two hours after ingestion with most reaching the host liver within six days of excystment. The flukes then migrate within the liver for approximately five to six weeks while feeding on the liver tissue directly. Eventually the flukes enter the bile ducts and attain sexual maturity. The flukes then lay eggs, which are passed through the faeces. The pre-patent period spans from the period the host ingests metacercariae, to the first visible eggs appearing in the faeces. The length of the prepatent period varies depending on the temperature, definitive host and the infection intensity based on the number of the adult flukes in the liver (Chen & Mott, 1990; Mas-Coma and Bargues, 1997).

II. The transit between definitive mammal host and intermediate snail host encompasses the long resistance phase of the egg and the short active phase of miracidium. After the eggs have been eliminated from the definitive host through the faeces, they can remain viable for several months until they reach freshwater with suitable physio-chemical characteristics (Lapage, 1968). The eggs develop into miracidia, under a temperature of 15-25°C, between 9 and 21 days with photo-stimulation. The miracidium moves against gravity towards a light source and swims rapidly after hatching until it finds an appropriate intermediate host snail (Mas-Coma and Bargues, 1997).

III. Upon contact with the host snail, the miracidium penetrates into the snail. The miracidium then develops into a sporocyst and then rediae (Carrique-Mas and Bryant, 2013). The redial development pattern is universal in the lymnaeid host snails with mono-miracidial infection resulting in three to four redial generations. Rediae then develop into cercariae within 6-7 weeks at 20-25°C. The cercariae are then shed into freshwater sources with temperatures varying between 9 and 26°C and independent of light stimulation (Mas-Coma and Bargues, 1997; Valero *et al.*, 2009).

IV. After shedding, the cercariae will swim for approximately an hour until they come across solid support in the form of leaves of water plants and then shed their tails and rapidly encyst and become metacercariae which become infective within 24 hours of encystment (Mas-Coma and Bargues, 1997).

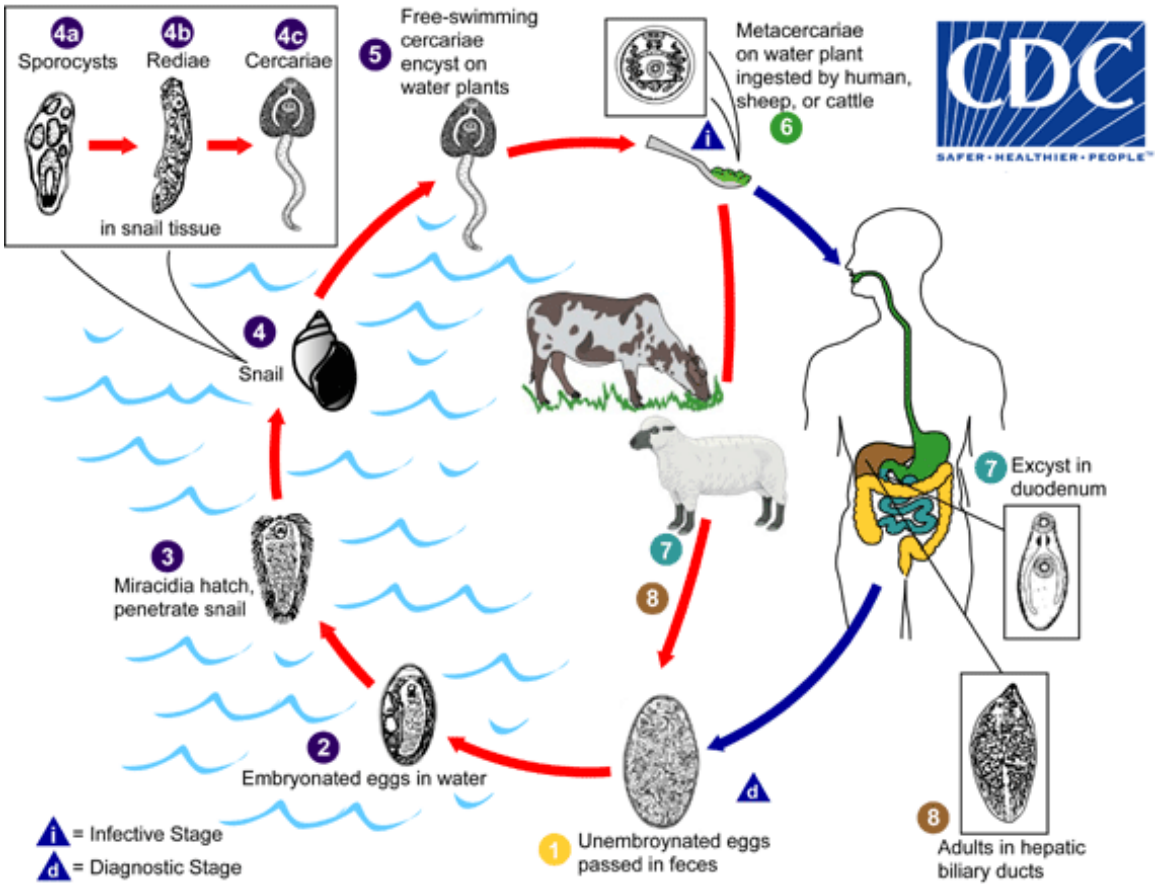

Figure 1: Life cycle of *Fasciola* spp. The developing eggs are secreted in the bile ducts and in the faeces (1). The eggs then develop and embryonate in water (2), miracidia are then released from the eggs (3) and the miracidia infect a compatible snail intermediate host (4). Ideal host snails may include the genera *Lymnaea*, *Galba*, *Fossaria* and *Pseudosuccinea*. The parasites then go through three developmental stages within the snail (sporocysts (4a), rediae (4b), and cercariae (4c). The resultant cercariae are released from the snail (5) and then encyst on solid surfaces such as aquatic plants and become metacercariae. The definitive mammalian hosts become infected upon by ingestion of vegetation containing metacercariae with the most common plants ingested by humans being watercress (6). After ingestion, the metacercariae undergo excystation in the duodenum (7) and migrate through the intestinal wall, the peritoneal cavity, and the parenchyma of the liver into the bile ducts. They then develop into typical adult flukes (8) (Cdc.gov, 2013).

2.4 Symptoms of infection

In most cases, the observed signs and symptoms of infection of the host by the fasciolid flukes show relation to the locality of the adult worms within the host (Kostadinova and Pérez-del-Olmo, 2014). Infection occurs in two distinct phases unlike the other hermaphroditic parasites. The initial phase of the disease may occur in the initial six to nine weeks of infection where the larvae migrate through the host liver. In the second phase, the flukes migrate to the bile ducts (Rojas *et al.,* 2014). Some of the symptoms shown by patients with acute clinical syndrome are prolonged fever, pain in the right hypochondrium, inflammation and localized destruction of liver cells, and in some cases hepatomegaly, asthenia, and urticarial (Rana *et al.,* 2007; Keiser and Utzinger, 2009). This period is also characterized by the occurrence of eosinophilia. Infection is typically acute and thus after the flukes enter the bile ducts, the symptoms normally subside and then disappear completely. Chronic biliary tract disease may occur in animals with heavy worm burden but rarely occurs in humans and is usually a secondary finding of surgery or autopsies when reported. Once the flukes migrate into the bile ducts, hosts may experience inflammation as a result of toxic secretions as well as mechanical irritation (Chen and Mott, 1990; Mas-Coma and Bargues, 1997). Autopsies and experimental models both show that the severity of the disease shares a direct relationship with the infection intensity and may also result in fibrosis (Doughty, 1996).

2.5 Diagnosis

Fascioliasis is diagnosed through examination of fecal/stool samples under a microscope to detect ova with an inconspicuous operculum (Cuomo *et al.,* 2009). Diagnostic methods for human fascioliasis currently involve the use of direct parasitological techniques, indirect immunological tests and other non-invasive techniques. Other non-invasive diagnostic techniques used are ultrasound, radiology, radioisotope scanning, computer tomography and magnetic resonance (Esteban *et al.,* 1998; Hillyer, 1999; Lukambagire *et al.,* 2015). Recently generated biological data shows emphasis for the necessity of developing quantitative coprological data analyses for utility in surveys focusing on epidemiology, as well as in post-treatment monitoring programs (Lukambagire *et al.,* 2015) for better control of the disease. Besides the use of eggs in coprological analyses, other more invasive techniques can be used to find adult flukes and eggs in other parts of the host. Such techniques include but are not

limited to extraction of duodenal fluid; various surgeries such as laparotomy, cholecystectomy, and sphincterotomy; duodenal and biliary aspirates as well as the histological examination of the liver and/or other organ biopsy materials. Studies show that serological, intradermal and specific coprologic antigen tests have recently been used in differentiation of the species of *Fasciola* in both field and clinical studies (Mas-Coma *et al.,* 2005; Lukambagire *et al.,* 2015).

Various serological tests, which involve detecting circulating antibodies, have been used in diagnosis of human fascioliasis (Keiser *et al.*, 2010). However, there is a limited number of techniques which are applicable in detecting circulating antigens and immune complexes. Serological techniques have also been recently utilized in monitoring the post-treatment evolution of the parasite. Immunological diagnostic techniques which take advantage of the recombinant *F. hepatica* cathepsin L 1cystine proteinase, the purified cathepsin L cystine proteinase, and cathepsin L-ELISA have been applied in the diagnosis especially in the pre-patent or acute phase of disease (O'Neil *et al.,* 1999; Santana *et al.,* 2013). These immunological techniques do however have the distinct advantages over standard techniques as they can be used during all stages of the disease, with emphasis on the acute phase. In other cases, where coprological examinations are inappropriate, immunological techniques are also advantageous (Keiser *et al.,* 2010). A disadvantage of immunological techniques is possibility of poor sensitivity and specificity (Santana *et al.,* 2013).

2.6 Treatment and control

2.6.1 Flukicides

For most of the intestinal helminth infections, safe and effective drugs are available (Panic *et al.,* 2014). The World Health Organization (WHO) coordinates periodic deworming programs for populations which are considered to be at risk of infection. This preventive chemotherapy has been the main strategy in control of major helminthiasis (Greter *et al.,* 2016). A variety of fasciolicides recommended for livestock are available and in use for the treatment of fasciolosis and these include triclabendazole, albendazole, oxyclozanide, clorsulon, nitroxynil, rafoxanide and oxfendazole (Fairweather, 2011; Villegas *et al.,* 2012). For fascioliasis in humans, triclabendazole is currently the recommended treatment (Keiser *et al.,* 2005). Triclabendazole has been shown to be the most effective flukicide applicable to the immature

and adult stages of the parasite and due to this, has been the default treatment course for acute fascioliasis. Studies have shown an increase in resistance by flukes to triclabendazole (Fairweather, 2011). Although efforts have been made to develop novel drugs which are more efficient against immature flukes, current results have not been conclusive. Research into efficacy of combining compounds such as clorsulon (2.0 mg/kg) and nitroxynil (10.2 mg/kg) has shown an efficiency of 95% against two-week-old flukes and 99% against four-week-old flukes (Hutchinson *et al.,* 2009).

2.6.1.1 Limitations of use of flukicides

In parts of Africa, mobile pastoralism is a highly used livestock dependent lifestyle as a result of scarcity of grazing land. This lifestyle is mostly driven by the constant search for water and ideal pastures for livestock (Krätli and Schareika, 2010). Camels, cattle, goats and sheep are often kept as sources of milk and meat for consumption; and are also quite often used in trade. The livelihood of the people who adopt this lifestyle is directly dependent on the health and the productivity of the livestock and this makes animal health a major point of concern (Bechir *et al.,* 2012). In developing countries with limited resources, health systems often do not have adequate governmental funding and qualified health or veterinary personnel with exception to upper-income areas (Sheik-Mohamed and Velema, 1999; Zinsstag *et al.,* 2005; Obrist *et al.,* 2007). In addition, official health providers do not cover a large amount of people or livestock. Medical and veterinary centers in rural areas often lack basic infrastructure, clean water, support staff. In countries like Chad, the private sector has had to develop business-oriented health services to cover the short fall. This has led to the development of an informal drug market with sales persons lacking training in most cases. Drugs purchased from these sales people often do not accompany guidance, drug information and documentation. Despite this, the industry has grown due to lack of purchase control and the inexpensiveness of the unofficial drugs (Gauthier and Wane, 2011; Djimouko and Mbairo, 2014). Advances in diagnostic techniques for human fascioliasis have however allowed for more time and labor efficient diagnosis especially in cases where the physicians and health personnel may not be aware of infection (Mas-Coma *et al.,* 2014; Ashrafi *et al.,* 2014).

Another major limit in cattle, with emphasis on dairy cows, is the differences in legislation which regulates the drug licensing between different countries. In Switzerland for example, only one clorsulon formulation has been authorized (Ivomec®Plus, Biokema SA), which can

only be administered to non-lactating animals. There are no registered nitroxynil drugs and instead, triclabendazole which has a withdrawal time of 12 days for milk (Endex®19.5%, Novartis AG) is used for dairy cows. In Germany however, triclabendazole is not authorized for use in cattle which are lactating. Due to lack of international standardization and consideration of individual epidemiological situation, it becomes the responsibility of the practitioner to optimize the drug administration. Additionally, triclabendazole is not registered for use in many countries, and is therefore not always available for the treatment of human fascioliasis (Keiser *et al.,* 2005).

2.6.1.2 Alternative methods of control

One of the main methods for control of fascioliasis is control of intermediate host snail populations below a threshold level (Jaiswal and Singh, 2009; Sunita and Singh, 2011). The freshwater snails are however, very important to the aquatic ecosystems and thus eliminating them would disrupt the ecosystem. Different plants derived component have been shown to be effective as molluscicides against the snail *Lymnaea acuminata* (Singh *et al.,* 1995; Singh *et al.,* 1997; Kumar and Singh, 2006). These plant derivatives such as citral, ferulic acid, umbelliferone, azadirachtin, and allicin were tested in combinations of equal parts against *Fasciola* larva in vivo (Sunita and Singh, 2011). Should these active plant derivatives be effective against the larvae of *Fasciola* flukes i.e. redia, cercaria, a binary combination with the plant derived components at sub-lethal concentrations within the snail could reduce the rate of infection without severely disrupting the ecosystem by eliminating the snail Jaiswal and Singh, 2009; Sunita and Singh, 2011).

2.6.1.3 Repurposed drugs

Another proposed treatment besides traditional flukicides is the use of antimalarials as a repurposed drug. Research into the repurposing of antimalarials has been based on the similarity of the blood-feeding characteristics of *Schistoscoma* spp., liver flukes and *Plasmodium* spp. If antimalarials target the heme degradation pathway, they could be useful against flukes which use the same pathway (Toh *et al.,* 2010).]. Although repurposing antimalarials has been shown to be effective even against non blood-feeding flukes (Ingram *et al.,* 2012), caution is essential due to the potential for development of resistance. However, malaria and schistosome infections usually overlap geographically, indicating the benefit of wide use of antimalarials in co-infected patients (Keiser and Utzinger, 2012).

2.7 Intermediate host

The freshwater snails under Lymnaeidae have been shown to be the intermediate hosts of both *F. hepatica* and *F. gigantica* (Bargues *et al.,* 2001). Besides planorbids, lymnaeid snails are also involved in a number of other helminth life cycles. They serve as intermediate hosts for numerous digenean trematode species and certain nematodes (Bargues *et al.,* 2001). Lymnaeid snails are involved in the life cycles of more than 71 trematode species, derived from 13 different families (Correa *et al.,* 2010). The members of these families use birds and both domesticated and sylvatic mammalian definitive hosts; however, these are not inclusive of the digeneans trematodes affecting lower vertebrates (Brown, 1978; Bargues *et al.,* 2001). The trematode species transmitted by lymnaeid snails is largely dependent on parasite–host specificity, the geographic region and local ecological characteristics (Adam and Lewis, 1993; Esch and Fernandez, 1993). The trematode-snail specificity can affect infectivity, compatibility, host finding, recognition and attraction, susceptibility, resistance, immunity, phylogeny and genetic variation (Sapp and Loker, 2000; Mas-Coma *et al.,* 2009). Lymnaeids transmit several trematode species which have immense medical and veterinary importance hence the great interest in these freshwater snails. Over the last few decades, an increase in the study of lymnaeids has been observed due to the detection of true human endemics, of varying prevalence and intensities. Studies have shown promise in the use of control of lymnaeid snails in combating parasites which they harbour (Jaiswal and Singh, 2009; Sunita and Singh, 2011), however currently knowledge is still limited. It is evident from recent studies that human fascioliasis should be considered on the list of important human disease caused by parasites and should not keep its current standing as a secondary zoonotic disease (Mas-Coma *et al..,* 1999; Mas-Coma and Bargues, 1997).

2.8 Impact of fascioliasis

Facioliosis undoubtedly has a global socio-economic impact. Some of the loses which can be attributed to this disease in livestock include loss of weight, reduction in productivity and draught capacity, diminished fertility and milk production; with the financial burden being losses exceeding US$3.2 billion annually (Spithill and Dalton 1998, Bernardo *et al.* 2011).

The emergence and re-emergence of human facioliosis has highlighted its importance as a zoonosis due to increased reports of human cases caused by both *Fasciola* species and the hybrid form (Schweizer *et al.,* 2005). Animal fascioliasis is known to be of great interest in veterinary field across the globe, with human fascioliasis being regarded as a secondary disease prior to the 90's. Its importance, however, increased in the 90's due to development of human epidemics and an increased number of patient reports (Chen and Mott, 1990; Ashrafi *et al.,* 2014). Various studies in pathology of the flukes showed that human infection is of serious health concern in the acute phase as well during the very long chronic phase (Ashrafi *et al.,* 2014). Due to the differences in the epidemiology and transmission of the disease observed in areas where human fascioliasis is endemic, diagnosis is difficult (Mas-Coma *et al.,* 2014). Fasciolids have also shown the capacity to induce immunosuppression and immunomodulation in both the acute and chronic phases of infection. This may result in hosts being infected, incidentally, with other parasites in areas exhibiting frequent cases of human fascioliasis (Ashrafi *et al.,* 2014). Studies have shown that changes in climate and environmental factors, which are resultant of human activity, have a significant effect on the human infection (Mas-Coma *et al.,* 2009; Afstan *et al.,* 2014). Another major factor which has resulted in increased reports of food-borne parasite infections has been an increasing demand for exotic and raw food across world markets (Mas-Coma *et al.,* 2014). The capacity of globalization to effect the distribution of food-borne parasites has only recently been taken into account and shown to be a hurdle for veterinarians, researchers and all parties involved with food and health safety (Broglia and Kapel, 2011; Robertsons *et al.,* 2015). The increase in travel between rural and urbanized areas as well as rural food traditions, including sylvatic vegetables, increasingly being imported to urban areas additionally demonstrates the distribution of *Fasciola* as a result of globalization (Ashrafi *et al.,* 2006; Mas-Coma *et al.,* 2014). During the metacercarial stage of the *Fasciola* life cycle, the metacercariae can remain viable and infective for up to 48 weeks. This allows the metacercariae, and in essence, the fasciolids, to remain viable for extended periods of time such as those observed in intercontinental travel (Ashrafi *et al.,* 2014). In light of research elucidating the increased importance and epidemiological significant of fascioliasis, the World Health Organization was compelled to include fascioliasis in the list of important human diseases and in the recent 2015-2020 roadmap for the control of neglected tropical diseases.

Even in the face of such compelling scenarios, fascioliasis is still not considered as being of elevated significance in Travel Medicine (Mas-Coma and Bargues, 2009) as a result of the following factors: (a) travelers are not legally obliged to declare the disease and hence most diagnoses are not reported in literature; (b) reports in travellers or migrants have not been broadly distributed through literature on the topic; (c) all human infections were considered to be accidental in rural areas which showed frequent infections; and (d) the global distribution of the disease in livestock resulted in an oversight in the risk for spread of the disease (Mas-Coma *et al.,* 2005; Ashrafi *et al.,* 2014). Studies have shown changes in the epidemiology of the disease as well as an increase in observed of human endemic areas thus the importance of fascioliasis in Travel Medicine should be significantly increased (Mas-Coma *et al.,* 2005; Mas-Coma and Bargues, 2009).

2.9 Human fascioliasis case studies in South Africa

Fasciola is known to be globally distributed, with human fascioliasis showing the widest distribution of any vector-borne disease (Mas-Coma *et al.,* 2003). Despite this, there are only three reported cases from South Africa, with the last report occurring in 1964 (Black *et al.,* 2013). The first two cases were reported in the Eden, Western Cape. In the first case, a woman from Natures Valley aged 73, observed symptoms which included an ongoing fever which lasted two weeks, shivering, discomfort, severe weight loss and dry coughs. Initial reports showed that the patient had no conclusive infection (Black *et al.,* 2013). Although she had travelled to Zambia and camped, 7 months prior to examination, she only consumed canned food which were purchased in South Africa, and filtered water. The woman also reported not eating other possible sources of infection such as fish or salads (Black *et al.,* 2013). After treatment, further analysis resulted in discovering that the female had ingested watercress purchased from a farm stall in Natures Valley in the three months before showing signs of disease. In the second case, a female chef aged 37 from Plettenberg Bay had shown the following showed a week-long history of breathing difficulty, central chest pains, irregular heartbeat, impaired linguistic ability and bouts of sudden weakness. The patient received treatment based on presented symptoms. Although the patient had never travelled out of the country, she had used watercress which was purchased locally in both professional and home food preparations thus presenting a window of infection (Black *et al.,* 2013). There have been no publications elucidating the details of the third case of human fascioliasis to date, however

it was reported in Johannesburg in 1964 (Scott and Irving, 1964) with symptoms indicating chronic infection (Black *et al.,* 2013).

2.10 Phylogenetic studies of *Fasciola* using molecular techniques

Although molecular markers have been successfully applied when studying genetic characteristics of fasciolids, two factors must be taken into account: (i) *F. hepatica* and *F. gigantica* are closely related with recent evolutionary divergence, and (ii) their current geographical distribution is resultant of prehistoric and some recent spreading events (Mas-Coma *et al.,* 2009). Due to this, molecular markers used to study Fasciolids must be able to differentiate between very closely related species. The Nad1 and Cox1 genes have been widely used in genetic analysis of the *Fasciola* spp. (Hashimoto *et al.,* 1997; Semyenova *et al.,* 2006; Teofanova et al *et al.,* 2011). These genes display comprehensive information which allows researchers to define polymorphic sites as molecular markers for use in phylogenetic as well as population genetic diversity studies. Sequence analysis of the Cox1 gene has been used to elucidate the taxonomic state of Japanese *Fasciola* spp. (Hashimoto *et al.,* 1997). Comparative analysis with *F. hepatica* has shown a difference of approximately 25-28 nucleotides from Japanese *Fasciola* spp., while *F. gigantica* differs only with 4-5 nucleotides. Inferences using the Nad1 and Cox1 gene sequences have shown to be useful at population level through studies of the Eastern European and Western Asian liver fluke populations showing 13 Nad1 and 10 Cox1 haplotypes (Semyenova *et al.,* 2006). These mitochondrial genes have also been useful in elucidating the phylogenetic relationship between *Fasciola* species and hybrids of the two species (Itagaki and Tsutsumi, 1998; Itagaki *et al.,* 1998, 2001; Peng *et al.,* 2009).

2.11 Other molecular markers

Phylogeny can be defined as the history of descent of a specific group of taxa, like species, from their common ancestors. This is inclusive of the order of branching between taxa, as well as the relative times of divergence between the taxonomic groups of interest. A more classical method of studying and inferring the phylogenetic relationships between taxa is

morphological studies of specimens (Brown, 2002; Patwardhan *et al.,* 2014). These studies however often require expert taxonomist and can be made difficult by cryptic species, which may have underlying differences not shown morphologically. The diversity of species may not only be phenotypic or morphological, but may be ultra-structural, biochemical or even variation in molecular features of the species. Advances in molecular techniques and/or technology over the last few decades have allowed for application of nucleic acid sequencing techniques to alleviate some of the issues and challenges faced by scientists with phylogenetic studies (Patwardhan *et al.,* 2014).

2.11.1 Molecular markers

As discussed above, diversity and variation between and within taxa can be seen beneath the phenotypic differences. Heritable and detectable variability can be studied through analysis of nucleic acid sequence with particular interest to DNA (Patwardhan *et al.,* 2014). By comparing homologous protein and DNA sequences, geneticist can infer relationships among organisms and/or genes in molecular phylogenetic studies (Patwardhan *et al.,* 2014). Divergence between sequences can be seen through differences in sequences which are resultant of molecular evolution caused by normal cell functions as well as interactions with the environment during the course of time (Liu and Cordes, 2004). Over time, DNA exhibits single nucleotide polymorphisms (SNPs), insertions and deletions (Indels), inversions and rearrangements (Liu and Cordes, 2004; Patwardhan *et al.,* 2014). These mutations may occur at different rates based on the gene and it is essential to keep this in mind before any genetic studies. The mutation rates of genes are largely influenced by the amount of change/mutation a gene can undergo before loss of function occurs (Liu and Cordes, 2004; Patwardhan *et al.,* 2014)). When selecting molecular markers, it is essential to consider the following factors in order to ensure a comprehensive and statistically sound study: (i) the genotyping method should be as simple and cost efficient as possible, allowing generation of vast amount of genotyping data; (ii) information content, the dominance relationships, genetic independence and neutrality of the selected markers (Patwardhan *et al.,* 2014). Based on the type of information they provide, molecular markers can be divided into three categories: (i) the bi-allelic dominant markers, as in the case of RAPDs, AFLPs; (ii) the bi-allelic co-dominant markers, like RFLPs, SSCPs and (iii) the multi-allelic co-dominant markers, such as the microsatellites (Patwardhan *et al.,* 2014).

The following factors must be considered in the selection of ideal molecular markers: (i) single-copy gene such as mitochondrial and nuclear genes may be more informative than multiple-copy genes (Cruickshank, 2002); (ii) ease of sequence alignment, however this may be difficult due to variation in sequence length across taxa. In such cases, regions which may cause ambiguous alignments should be avoided and secondary structure information used (Gatesy *et al.,* 1993); (iii) the rate of substitution should be ideal in order to provide enough informative sites (Cruickshank, 2002); (iv) the primers should be available to amplify the marker without non-specific amplification and (v) inference of ancestry may be influenced negatively by an excess of base variation among taxa (Patwardhan *et al.,* 2014).

2.11.1.1 Nuclear ribosomal genes

A common choice of markers are nuclear ribosomal genes. Ribosomal RNA is ideal for analysis of phylogenies due to it being universal and its composition variable regions within highly conserved domains (Woese , 1990; Gillespie *et al.,* 2006). Ribosomes consist of two subunits; the small ribosomal subunit (SSU) and the large ribosomal subunit (LSU). The SSU contains single RNA classes i.e. 18S rRNA in eukaryotes and 16S rRNA in other taxa, while the LSU contains two RNA classes (the 5S and 23S rRNAs) in bacteria and Archaea; and three RNA species (the 5S, 5.8S and 25S/28S rRNAs) in most eukaryotes (Parsch *et al.,* 2000; Patwardhan *et al.,* 2014). The variable region of rRNA can be amplified using primers for the conserved regions as it lies between the conserved regions and this allows to differentiate organisms with a good measure of statistical validity (Patwardhan *et al.,* 2014).

2.11.1.2 Mitochondrial genes (mtDNA)

Mitochondrial DNA is non-recombinant and is only inherited maternally allowing scientists to trace maternal lineage (Patwardhan *et al.,* 2014). It also shows higher mutation rates than nuclear genome due to inefficient DNA repair mechanisms making it quite advantageous in genetic studies (Boore, 1999; Galtier et.al., 2009; Teofanova *et al.,* 2012). Mitochondrial gene order varies with the genes separated by large regions of noncoding DNA. mtDNA has become ideal in phylogenetic analysis and population genetic structuring due to the following factors: i) developments in techniques in isolation of mtDNA, ii) utility of restriction enzymes

in detection of nucleotide differences, iii) the advances in PCR technology and iv) viability of universal primers for PCR amplification of mtDNA (Patwardhan *et al.*, 2014).

2.11.1.2.1 Cytochrome oxidase I/II (COI/II)

The enzyme cytochrome c oxidase is largely recognized as the main protein used in the electron transport chain in both bacteria and mitochondria (Kress and Erickson, 2008; Patwardhan *et al.,* 2014). The COI and COII genes encode two of seven polypeptide subunits in the cytochrome c oxidase complex (Patwardhan *et al.,* 2014). The cytochrome c oxidase subunit I is a widely recognized as molecular marker and is applicable in species identification (Locke *et al.,* 2010; Krishnamurthy and Francis, 2012). It is also recognized as the ideal DNA barcode gene by GenBank and the Consortium for the Barcode of Life (CBOL) (Kress and Erickson, 2008).

2.12 Conclusion

Several studies have been conducted around the world, showing a wide distribution of the Fasciolid liver flukes (Spithill and Dalton 1998; Mas-Coma *et al.,* 2003). Literature shows an immense adaptive capability of the flukes and their intermediate host snails as evident from the wide range of geographic regions showing endemicity of the disease (Mas-Coma, 2004). Studies of epidemiological patterns as well as diversity of *Fasciola* are essential to allow efficient management and control of fascioliasis (Cwiklinski *et al.,* 2015).

CHAPTER 3: Phylogenetic analysis of *Fasciola* spp. from KwaZulu-Natal and Mpumalanga provinces in South Africa based on the CO1 mitochondrial marker

3.1 Abstract

Fasciola hepatica, *Fasciola gigantica* and intermediates are the main causative agents of fascioliasis in humans and livestock. Prior to development of control and management measures, geographical distribution and patterns of infections by the different *Fasciola* species must be considered. Due to difficulties in phenotypic differentiation of *Fasciola* spp., molecular markers have been used to determine fluke differentiation and description of phylogenetic patterns. The mitochondrial genome has been shown to be highly variable with the cytochrome oxidase I (CO1) gene showing utility as a DNA barcode and tool for phylogenetic analysis. In this study, the CO1 mtDNA marker was sequenced from 55 flukes collected from three abattoirs with catchment areas in the KwaZulu-Natal and Mpumalanga provinces of South Africa in order to identify and describe the genetic diversity of the flukes in cattle within the study regions. Based on the CO1 sequence analysis, *Fasciola hepatica* was shown to have a 100% prevalence in KwaZulu-Natal, 100% prevalence in the highveld of Mpumalanga and 76% prevalence in the lowveld (Belfast) of Mpumalanga. Two animals from the Belfast metapopulation were co-infected with both *F. gigantica* and *F. hepatica* and the phylogenetic relatedness of *F. gigantica* isolates were compared with those from the genbank including the intermediates. Twenty-two haplotypes were identified with eighteen novel haplotypes being unique to the isolates from South Africa. Two novel *F. gigantica* and sixteen *F. hepatica* haplotypes were identified from the study. Notably, only one haplotype was shared between the experimental *F. hepatica* isolates and the genbank isolates from China and Niger. *Fasciola gigantica* isolates from the study did not share any haplotypes with the genbank isolates from Zimbabwe, China and Niger, however, they showed close relation with only one mutational step between haplotypes. Within the study samples, a number of haplotypes were isolated to a few individuals indicating high diversity. The distribution of *Fasciola* spp., within the scope of this study, has been shown to be related to the distribution of the freshwater intermediate host snails, *Lymnaea spp,* as well as relative altitude of the localities in South Africa. Information provided by this study will serve as a starting in mapping the distribution of *F. gigantica* and *F. hepatica* in South Africa in order to design and monitor control programs for fascioliasis in humans and livestock.

Keywords: *Fasciola hepatica, Fasciola gigantica, mitochondrial markers (CO1), phylogeny, haplotypes, distribution*

3.2 Introduction

Fascioliasis is a food-borne parasitic disease with a near global distribution with dire socio-economic effects (Spithill and Dalton 1998; Mas-Coma *et al.,* 2003; Mas-Coma *et al.,* 2009). The disease occurs as a result of infection by digenean trematodes of the genus *Fasciola*. Several species have been described under the genus, with the taxonomic standing still under scrutiny and debate. However, the main causative agents of fascioliasis are reported to be *Fasciola hepatica* and *Fasciola gigantica* (Caple *et al.,* 1978; Ai *et al.,* 2011). The two trematode species are known to infect mainly humans and ruminants resulting in losses in livestock production, fertility and resistance to draught for the later (Keiser and Utzinger, 2007; Bernardo *et al.* 2011). Economic losses in livestock have been reported to exceed three billion United States dollars annually with the zoonotic aspect of the disease also making it a concern in public health (Spithill and Dalton 1998; Mas-Coma *et al.*, 2009).

Over the last few decades, reports of fascioliasis have increased resulting in the increasing need for taxonomic clarity as well as differentiation of the two fasciolid species. Morphological techniques, primarily used in diagnosis, take basis on differentiation of morphological characteristics of eggs and the adult fluke in order to identify the species under study (Mas-Coma *et al.,* 2005; Cuomo *et al.,* 2009; Thanh, 2012). However, these techniques have shown limited sensitivity and specificity as they require skilled taxonomists, which are currently in decline. There have also been several reports of hybrids of the two species which may not be morphologically differentiated from the main species (Itagaki and Tsutsumi, 1998; Itagaki *et al.,* 1998; Schweizer *et al.,* 2005). With advances in molecular techniques, application of molecular markers in species identification and genetic characterization has become popular in studying *Fasciola* isolates (Elliot *et al.,* 2014; Robles-Pérez *et al.,* 2014; Cwiklinski *et al.,* 2015; Mucheka *et al.,* 2015).

Several studies have shown the utility of nuclear ribosomal markers such as ITS 1 and ITS 2 in species identification, however, mitochondrial markers are highly variable and can resolve taxonomic patterns of more closely related species and/or populations (Patwardhan *et al.,* 2014; Mucheka *et al.,* 2015). The cytochrome c oxidase subunit 1 (CO1) and NADH dehydrogenase subunit 1 (Nad1) have been used as molecular markers for elucidating the phylogenetic relationships of *Fasciola* isolates as well as describing, to an extent, the genetic diversity of fluke populations (Ai *et al.,* 2011; Itagaki *et al.,* 1998; Hashimoto *et al.,* 1997;

Peng *et al.*, 2009; Semyenova *et al.*, 2006; Mucheka *et al.*, 2015). The CO1 marker is widely regarded as an efficient DNA barcode and thus is commonly used in species identification and differentiation of *Fasciola* spp. in recent studies in South Africa and Zimbabwe (Mucheka *et al.*, 2015).

Against this background, the present study was aimed at identifying *Fasciola* spp. isolates collected from cattle in KwaZulu-Natal and Mpumalanga provinces using the (mtDNA) CO1 region and also examine and inter-species genetic diversity among the isolates using CO1 haplotypes.

3.3 Materials and methods

3.3.1 Study areas and collection of samples

A total of fifty-five flukes were collected from cattle abattoirs located in KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces (Figure 2) respectively. The abattoirs served as catchment areas for cattle slaughter in their respective areas and the exact location of origin of each animal was not determined. Twenty-one flukes were collected from a sale in the Swartberg and Lion's river in the Underberg area of KZN (each fluke was unique to one animal), seventeen were collected from the Barberton abattoir, Belfast, (MP) (two to three flukes collected per animal) and seventeen from the Mpumalanga Highveld (MP) (one fluke from each animal). The Underberg region of KZN has a warm and cold season with an average temperature of 14.1 °C. The average annual rainfal is 985 mm with the altitude being 1540m above sea level (Climate-Data, 2018a). The climate and weather patterns in the Mpumalanga province are dependent on the topography of the spcific region analyzed. In Belfast, which is in the lowveld, the climate is warm and temperate. The average temperature in Belfast is 13.2 °C and annual precipitation is 835 mm on average. In the highveld, the average annual temperature is 15.5 °C with the rainfall averaging 683 mm. The altitude noted in the Mpumalanga province ranges from 1500 m to more than 2000 m above sea level. A decrease in temperature is noted from east to west with the increase in altitude (Climate-Data, 2018b).

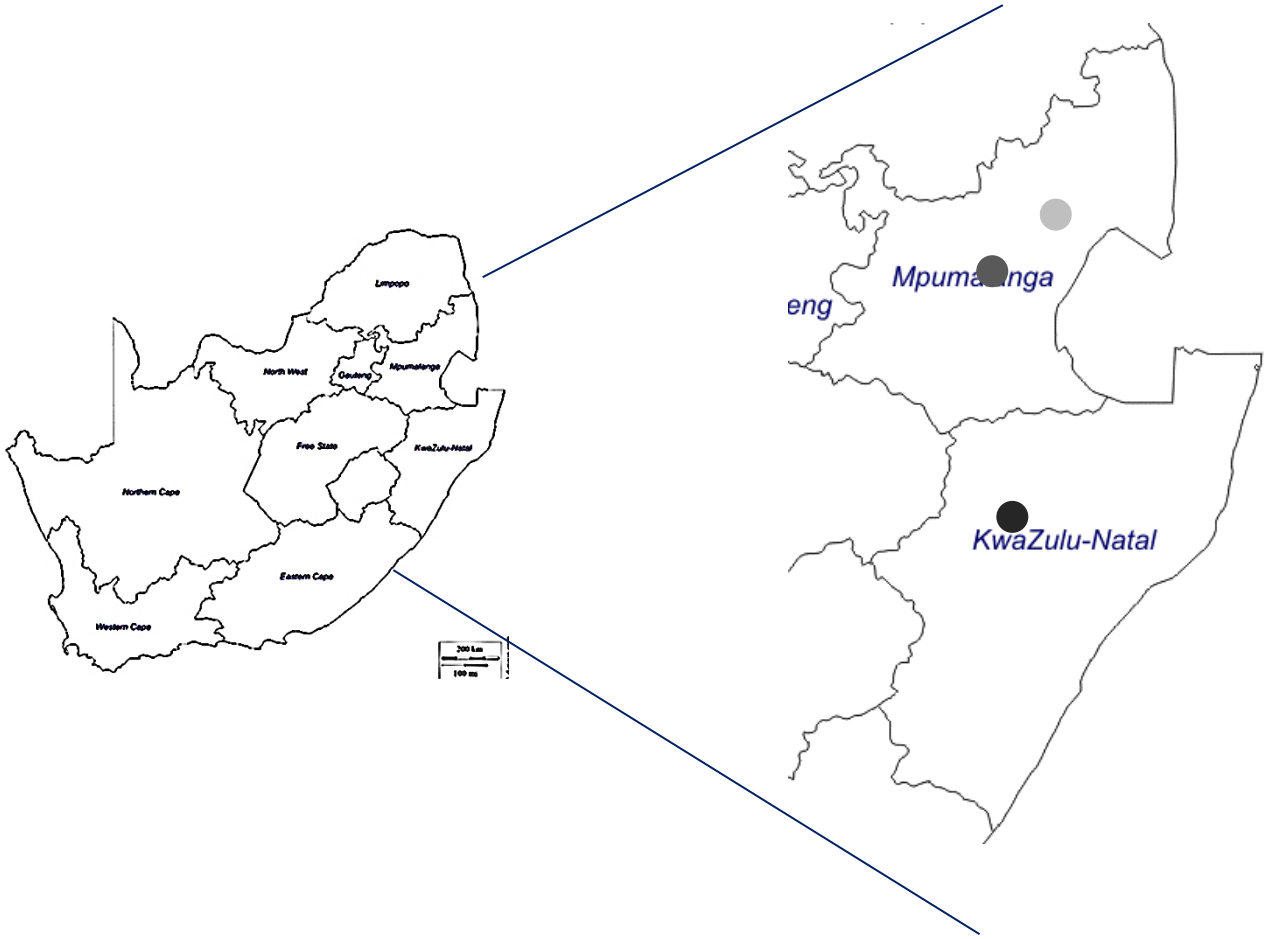

Figure 2. Localities were *Fasciola* spp. isolates were collected for the study. Dark grey= Underberg sale (KZN), grey=Highveld Mpumalanga, light grey=Barberton abattoir**.**

3.3.2 DNA extraction, amplification and sequencing

The genomic DNA was isolated from tissue from the posterior-end of the fluke using the phenol-chloroform method. All DNA was stored at -20^{o}C until further analysis. PCR amplification of the partial CO1 region was amplified using the primers: FHCO1 (forward: 5′-

TTGGTTTTTTGGGCATCCT-3′) and FHCO1 (reverse: 5′ -
AGGCCACCACCAAATAAAAGA3′) (Mucheka *et. al.*, 2015).
All PCR reactions were performed in 25 µL volumes with each reaction containing 12.5 µL of DreamTaq PCR Master Mix (2X), 0.5 µL per primer of the forward and reverse primers, 10,5 µL of nuclease free water and 1 uL of template DNA. PCR was performed in a thermocycler (BIORAD) under the following cycling conditions: initial denaturation at 95°C for 3 min, followed by 48 cycles of denaturation at 95°C for 30sec, annealing at 59°C for 30 sec and 72 °C for 1 minute and a final elongation step of 10 minutes at 72°C, with a 10°C hold.

PCR products were run on a 1% (w/v) agarose gel, which was stained with ethidium bromide. On each gel, a molecular weight marker of 100-1000 bp (O'GeneRuler, Fermentas, South Africa) was used to determine the size of any visible bands. The gel was viewed using the ChemiDoc™ MP Imaging system (BIO RAD) to confirm amplification.

The unpurified PCR products were subsequently sent to the Central Analytical Facilities (CAF), Stellenbosch University (South Africa) for sequencing using next generation sanger.

3.3.3 Sequence Analysis

All sequences were edited using BioEdit v7.2.5 (Hall, 1999) with sequence identity being confirmed using the nucleotide Basic Local Alignment Search Tool (BLASTn) tool in NCBI (National Centre for Biotechnology; www.ncbi.nlm.nih.gov/). Sequence alignment was performed using the MUSCLE function in Mega 7. DnaSP v5 (Librado and Rozas, 2009) was used to estimate the population diversity and demography. The phylogenetic relationships among the populations were inferred using MrBayes v3.2.6 (Ronquist *et al.*, 2012) with the following parameters; number of generations=200 000, print frequency=1000 sample frequency=10, number of Markhov chains=4 for Bayesian Inference (BI). The haplotype network was constructed using Network 5 (Bandelt *et al.*, 1999).

3.4 Results

3.4.1 Fluke identification

Based on the CO1 sequence identities, isolates from the Underberg region of KwaZulu-Natal were identified as *F. hepatica*, with a prevalence of 100%, and the same results were observed

with the isolates from the Mpumalanga highveld. Thirteen isolates were identified as *F. hepatica* (76 %) and four isolates as *F. gigantica* (24 %) from the Barberton abattoir in the Belfast area of Mpumalanga (Table 4). Two animals showed co-infection with both *F. hepatica* and *F. gigantica* (Table 1).

Table 1: Identity of *Fasciola* spp. isolates from three locations in KwaZulu-Natal and Mpumalanga provinces of South Africa based on CO1 mitochodrial marker sequences.

Study Province	**Origin of cattle**	**Number of isolates**	**Identity of isolates**		**% Prevalence of *F. hepatica***	**No. of cattle with co-infection**
			F. hepatica	***F. gigantica***		
KZN	Underberg	21	21	0	100	0
Mpumalanga	Highveld	17	17	0	100	0
Mpumalanga	Paardeplaas, Belfast	17	13	4	76	2

3.4.2 Sequence variation and diversity

The aligned KZN sequences showed a sequence conservation of 0.57, indicating moderate sequence variation although conservation is high. The nucleotide diversity was estimated to be 0.086 indicating low probability of difference in random sequences. Tajima's test was performed to test deviation of the sequences from the mutation drift equilibrium. The test yielded a Tajima's D statistic of -0.75339, which is indicative of negative selection; however, the results were not statistically significant ($P > 0.10$). The Belfast (MP) sequences showed a sequence conservation of 0.641 indicating high sequence conservation. The nucleotide diversity was estimated to be 0.069 also indicating low probability of random sequence difference. Tajima's test showed a D value of -0.84641, however, it was not statistically significant ($P > 0.10$). Highveld (MP) sequences showed a sequence conservation of 0.457 indicating high sequence variation than conservation. The nucleotide diversity was estimated to be 0.109. A Tajima's D of -0.608 also indicative of negative selection although not significant ($P >0.10$).

The total aligned sequences showed a sequence conservation of 0.472 indicating moderate sequence variation with higher variable sites than conserved sites. The nucleotide diversity was estimated to be 0.082 showing low probability of random sequence difference. Tajima's D was estimated to be -1.100 and not statistically significant (P > 0.05) (Table 2).

Table 2: Sequence diversity of CO1 in *Fasciola* isolates from the Kwa-Zulu Natal and Mpumalanga provinces.

Population	**Number of haplotypes**	**Haplotype diversity (hd)**	**Nucleotide diversity (Pi)**	**Tajima's D**	**Sequence conservation**
Kwa-Zulu Natal(KZN)	13	0.8880	0.08632	-0.75339	0.573
Mpumalanga highveld	9	0.7328	0.10911	-0.60777	0.457
Belfast	6	0.7378	0.06901	-0.84641	0.641
Combined	22	0.8957	0.08249	-1.10025	0.472

3.4.3 Genetic distances between haplotypes

The genetic p-distances between the F. hepatica isolates ranged from 0.01-0.28, while a range of 0.02-0.04. Interestingly, the Highveld haplotypes (H_15, H_16, H_17, H_20) showed a relatively higher genetic distance to the rest of the F hepatica haplotypes with a range from 0.09-0.28 (shaded grey in Table 3). Genetic distance between haplotypes identified within the two study species ranged from 0.07- 0.35 (Table 3).

Table 3: Estimates of genetic distances between haplotypes based on CO1 sequences of experimental and genbank *Fasciola* spp. isolates. Genetic p-distances are shown below the diagonal and the standard error(s) are shown above the diagonal.

Hap.	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8	H_9	H_10	H_11	H_14	H_15	H_16	H_17	H_18	H_19	H_20	H_12	H_13	H_21	H_22
H_1		0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.05
H_2	0.04		0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.05
H_3	0.11	0.10		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.04	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	0.06
H_4	0.04	0.03	0.10		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.02	0.03	0.02	0.01	0.04	0.02	0.02	0.02	0.05
H_5	0.03	0.02	0.09	0.03		0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.04	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.05
H_6	0.04	0.02	0.10	0.03	0.02		0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.05
H_7	0.05	0.06	0.13	0.07	0.05	0.05		0.01	0.01	0.01	0.01	0.01	0.04	0.02	0.03	0.02	0.01	0.04	0.02	0.02	0.02	0.06
H_8	0.03	0.03	0.10	0.03	0.02	0.02	0.05		0.01	0.01	0.01	0.01	0.04	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.05
H_9	0.03	0.02	0.09	0.02	0.01	0.02	0.05	0.02		0.01	0.01	0.01	0.04	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.05
H_10	0.03	0.03	0.10	0.03	0.02	0.02	0.05	0.03	0.01		0.01	0.01	0.04	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.05
H_11	0.04	0.04	0.11	0.04	0.02	0.03	0.06	0.03	0.02	0.02		0.01	0.04	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.05
H_14	0.03	0.03	0.10	0.03	0.02	0.02	0.05	0.02	0.01	0.02	0.02		0.04	0.02	0.03	0.02	0.01	0.03	0.02	0.02	0.02	0.05
H_15	0.23	0.26	0.25	0.29	0.25	0.26	0.20	0.25	0.27	0.26	0.25	0.25		0.03	0.03	0.04	0.04	0.05	0.05	0.05	0.05	0.12
H_16	0.09	0.11	0.17	0.13	0.11	0.10	0.07	0.10	0.11	0.10	0.11	0.10	0.14		0.02	0.02	0.02	0.04	0.03	0.03	0.03	0.07
H_17	0.16	0.18	0.16	0.19	0.17	0.17	0.14	0.17	0.18	0.18	0.16	0.16	0.13	0.12		0.03	0.03	0.04	0.04	0.04	0.04	0.09
H_18	0.11	0.12	0.14	0.13	0.11	0.12	0.09	0.11	0.11	0.10	0.11	0.11	0.22	0.10	0.15		0.02	0.03	0.03	0.03	0.03	0.07
H_19	0.06	0.05	0.09	0.06	0.05	0.05	0.07	0.05	0.05	0.05	0.06	0.05	0.22	0.09	0.14	0.08		0.03	0.02	0.02	0.02	0.05
H_20	0.22	0.21	0.20	0.23	0.21	0.21	0.23	0.21	0.22	0.21	0.21	0.21	0.28	0.24	0.23	0.16	0.16		0.05	0.04	0.05	0.08
H_12	0.10	0.08	0.16	0.07	0.09	0.09	0.12	0.09	0.08	0.09	0.09	0.09	0.35	0.18	0.25	0.20	0.13	0.30		0.01	0.00	0.04
H_13	0.12	0.09	0.17	0.09	0.10	0.10	0.14	0.11	0.09	0.10	0.10	0.10	0.34	0.20	0.26	0.19	0.13	0.26	0.02		0.01	0.05
H_21	0.12	0.09	0.17	0.08	0.10	0.10	0.13	0.10	0.09	0.10	0.10	0.10	0.36	0.20	0.26	0.21	0.14	0.31	0.02	0.04		0.04
H_22	0.35	0.31	0.39	0.30	0.32	0.33	0.36	0.33	0.31	0.32	0.33	0.33	0.62	0.44	0.51	0.44	0.36	0.51	0.28	0.31	0.27	

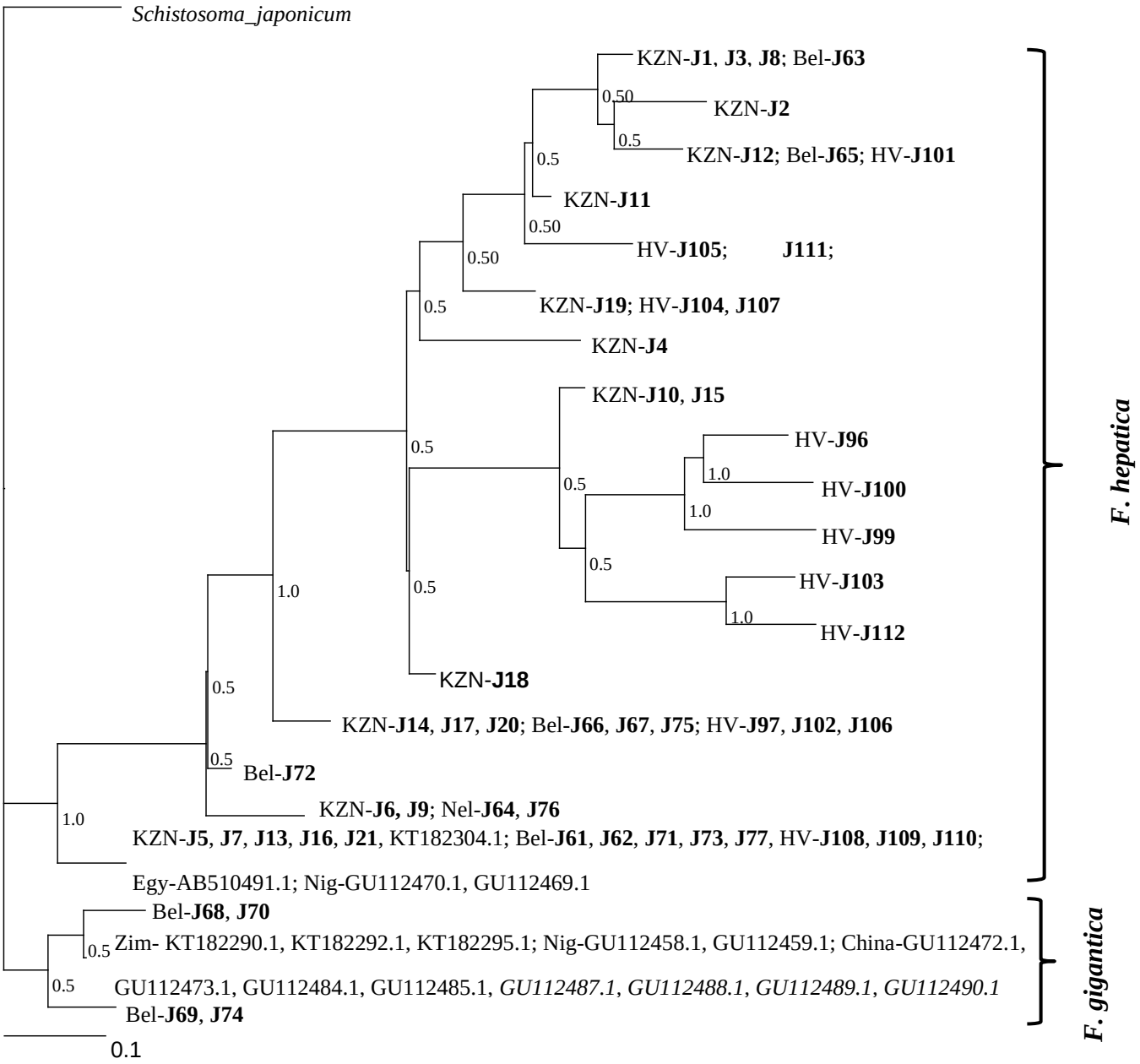

Figure 3: Phylogram based on haplotypes from CO1 sequences of *Fasciola* spp. populations from South Africa (KZN and Mpumalanga provinces) and genbank sequences using Bayesian inference. Experimental isolates are highlighted in bold and *Fasciola* spp. intermediates are shown in italics.

3.4.4 Phylogenetic analysis

The phylogenetic analysis based on the CO1 gene showed two main clades as shown in Fig. 3. The first clade constitutes *F. hepatica* based on identity of sequences from genbank and the current study samples which were well supported. The clade contained *F. hepatica* samples from all study sites and genbank sequences from Egypt, Niger and South Africa (Pietermaritzburg). The second clade constitutes *F. gigantica* based on genbank samples from Zimbabwe, Niger and China (including intermediates) and sequences from Belfast in our current study.

3.4.5 Haplotype distribution

Analysis of experimental and Genbank *Fasciola* spp. sequences showed twenty-two haplotypes with a relatively high diversity of 0.896. Haplotype H_4 was shared by *F. hepatica* sequences from KZN, Belfast, Highveld Mpumalanga, Niger and China (Fig. 3). Haplotype H_21 was shown to include *F. gigantica* sequences from China, Niger and Zimbabwe and notably did not include any of the *F. gigantica* study samples, which were isolated to haplotypes H_12 and H_13 as part of the haplogroup. Haplotype H_8 and H_9 were common amongst the localities within the scope of this study i.e. KZN and Mpumalanga while haplotypes H_2, H_3, H_7 and H_10 were unique to KZN. Haplotypes H_1 and H_5 were shared between KZN and Highveld (MP); and haplotype H_11 containing sequences from the Highveld and Lowveld (Belfast) of Mpumalanga. Haplotypes H_15 and H_20 contained sequences from the Highveld of Mpumalanga while haplotype H_14 was isolated to the Belfast locality and haplotyple H_6 was isolated to Zimbabwean isolates.

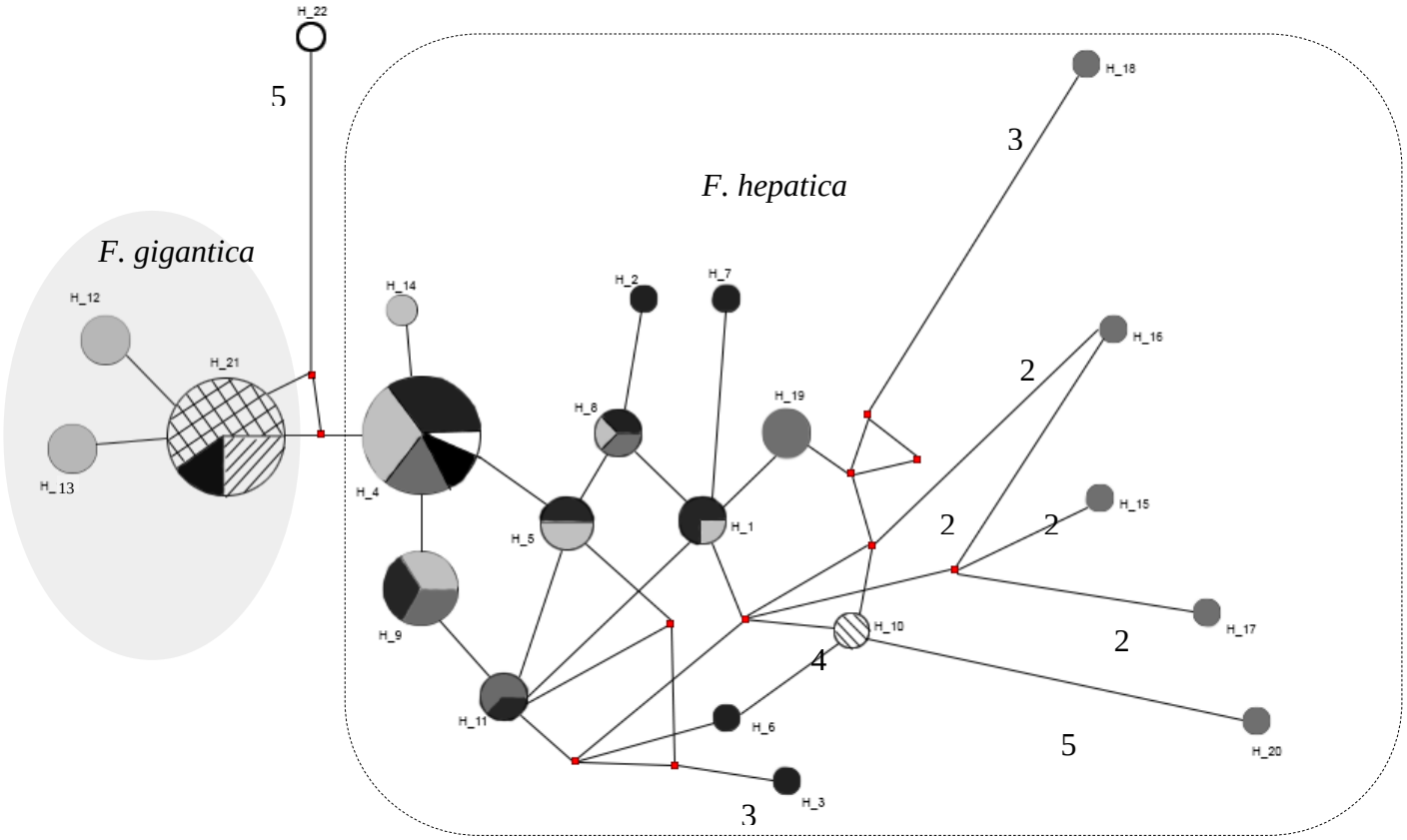

Figure 4: Haplotype network of *Fasciola* spp. isolates from the study and Genbank sequences. Haplotypes are represented by circles with the size of the circle indicating the number of sequences sharing that haplotype. The red squares represents median vectors. Numbers adjacent to haplotype links are the number of inferred mutational steps if more than one. Each colour is representative of the relevant geographical locality of the samples. Black fill = Niger, dark grey fill = Underberg (KwaZulu-Natal, South Africa), grey = Highveld (Mpumalanga, South Africa), light grey = Belfast (Mpumalanga, South Africa), white = Egypt, backward diagonal = Zimbabwe, diagonal cross = China, bold circle = outgroup.

3.5 Discussion

Phenotypic identification of *Fasciola* isolates is normally used although with difficulties in the diagnosis and clinical research (Ai *et al.,* 2011). Research has shown the necessity of molecular techniques in differentiation of *Fasciola* isolates and their utility in disease control and management. *Fasciola hepatica* and *F. gigantica* isolates were differentiated in this study with phylogenetic analysis of partial CO1 region showing separate clades of the *Fasciola* species. *Fasciola hepatica* was found to be prevalent in study localities in South Africa with 100% of isolates in KZN and Highveld Mpumalanga being *F. hepatica* as well as 76.5% of Belfast isolates being identified as *F. hepatica*. This study has shown similar results with respect to distribution of *Fasciola hepatica* in the KwaZulu-Natal and Mpumalanga provinces of South Africa (Mucheka *et al.,* 2015).

A major driving factor of the wide distribution is the dependence of the *Fasciola* life cycle on the fresh water snail intermediate host (Brown, 1978; Bargues *et al.,* 2001; Mas-coma *et al.,* 2009; Marquardt and Demaree Jr., 1985). Although several snail species are used by fasciolids, *Lymnaea* spp. are notably the primary intermediate host of *Fasciola* in South Africa. *Lymnaea (Galba) truncatula* currently noted as the preferred intermediate host of *Fasciola hepatica* in Europe, with studies showing its distribution across South Africa being scattered but showing higher prevalence in the KwaZulu-Natal and Mpumalanga regions as well as in the south of Western Cape province (de Kock *et al.,* 2003, Mucheka *et al.,* 2015). *Lymnaea (Pseudosuccinea) columella* is known to be more invasive and widely distributed in South Africa and is an intermediate host for both *F. hepatica* and *F. gigantica*. Results from this study shows an overlap in distribution of the lymnaeid species transmitting the two *Fasciola* species.

Lymnaea natalensis has been identified as an intermediate host for *Fasciola gigantica* (de Kock *et al.,* 1989) and hence its distribution in South Africa can be related to that of *F. gigantica.* It has been reported in the Mpumalanga province of South Africa, which could explain the presence of *F. gigantica* isolates in the Belfast region apart from the presence of *L. columella*.

The distribution of the snail intermediate hosts and the *Fasciola* species which they transmit, is dependent on several environmental factors which to a certain extent, are specific to the snail species. *Lymnaea truncatula* shows preference to the cooler areas and is most abundant in relatively high altitudes (Brown, 1980), while *L. natalensis* shows a wide distribution in lower altitudes. *Lymnaea columella* shares a similar distribution with *L. truncatula* and has

been shown to be highly invasive and adaptive as well. The present study showed high prevalence of *F. hepatica* in KwaZulu-Natal, Highveld and Belfast of Mpumalanga province. Studies have shown similar results (Mucheka *et al.*, 2015) giving an inclination to attributing the spread of *F. hepatica* in South Africa (within the study scope) to preferred habitats of the intermediate host snails *L. trancatula* and *L. columella*. *Fasciola gigantica* isolates were only found in the Belfast region of Mpumalanga, which could be due the area being a suitable habitat for *L. natalensis* and *L. columella*. An overlap of *Fasciola* spp. was noted in the Belfast region with two cattle showing infection with both species and further studies should be done to determine existence of intermediate forms of *Fasciola* as they have been reported in regions showing species overlap such as China (Ai *et al.,* 2011). No intermediates were identified in the current study, however, the *F. gigantica* isolates from Belfast showed close relationship with intermediates from China.

A link between environmental conditions and genetic diversity has been established based on Darwin's theory on adaptation to environmental stresses as well as geographical location (Walker *et al.,* 2011). The results of this study show low interspecific genetic diversity (π range between metapopulations = 0.06901-0.10911 and 0.07784 overall) between the flukes. This was comparatively higher than that observed by Ai *et al.* (2011) in a study of diversity of flukes, however their study consisted of isolates with a wider geographical distribution. Elliot *et al.* (2014) recorded lower diversity in *F. hepatica* isolates from Australia. The low nucleotide diversity suggests a recent population bottleneck or founder effects as the flukes are not indigenous to South Africa but have rather spread from Eurasia (Mas-Coma *et al.,* 2009). A relatively high haplotype diversity (hd = 0.8957) was also observed in this study which is comparatively similar to that noted by Mucheka *et al.* (2015) in a study of *Fasciola* isolates from South Africa and Zimbabwe. Notably, only one *F. hepatica* haplotype was shared between the study isolates and genbank derived isolates from South Africa (KZN), Niger and China. The shared haplotype suggest a phylogenetic relationship between the isolates. Propagated by the highly invasive *L. columella* and *L. truncatula* intermediate host snails, *F. hepatica* shows an expanding distribution globally. *Lymnaea columella* was first reported in South Africa in 1942 with studies showing rapid expansion of its distribution across sub-Saharan Africa (Appleton, 2003). *Fasciola gigantica* has been reported to be not as widely distributed as *F. hepatica*, however, its presence has been reported in South Africa (de Kock *et al.,* 1989; Mucheka *et al.,* 2015) unlike countries like Zimbabwe where it is predominat (Mucheka *et al.,* 2015). *Fasciola gigantica* isolates from this study did not share any haplotypes with the *F. gigantica* genbank isolates, however, they showed relation with only one mutational step between the haplotypes. The isolates forming part of the *F. gigantica*

haplogroup could share a distant maternal lineage. *Fasciola gigantica* is reported to have spread to Africa earlier than *F. hepatica* (Mas-Coma *et al.,* 2009) which could influence the rate of divergence of the populations isolated in South Africa due to the reported short time of haplotype change in fasciolids (Walker *et al.,* 2011). The high haplotype diversity is also indicative of a recent population expansion, which can be attributed to the ability of fasciolids to adapt to a wide range of environmental conditions (Mas-Coma, 2004; Mas-Coma *et al.,* 2005). Climate change as well as human activities have been cited as major contributory factors of the transmission and spread of fascioliasis (Mas-Coma *et al.,* 2009; Afshan *et al.,* 2014). Life cycles of both the liver flukes and their intermediate host snails, are highly dependent on geo-climatic parameters with emphasis on temperature, rainfall and altitude (Mas-Coma, 2004). Variations in climatic conditions can result in the variations in genetic diversity of the liver fluke between populations, displaying spatial and temporal separation (Walker *et al.,* 2011).

3.5 Conclusions

Although an increase in the importance of facioliasis as a zoonotic disease has been observed, the knowledge gap in the phylogenetic relationships as well as genetic diversity of *Fasciola* spp. is evident. This study has shown the presence of *F. hepatica* in both the KwaZulu-Natal and Mpumalanga provinces of South Africa, confirming a recent observation by Mucheka *et al.* (2015) with *F. gigantica* only observed in the lowveld of Mpumalanga province. *Fasciola hepatica* was shown to be prevalent in the two provinces studied and shows similarity to studies by Mucheka *et al.* (2015). A link between the distribution of *Fasciola* spp. and that of the lymnaeid intermediate host snails has been noted in literature (Mas-Coma *et al.,* 2009; Mucheka *et al.,* 2015) giving an inclination to attributing the fasciolid distribution to that of the snail intermediate hosts. The mitochondrial CO1 marker showed high genetic diversity between the study populations, however, further studies using more variable markers, such as microsatellites is required to further elucidate population genetic structuring. Future research should be focused on elucidating the phylogeography of *Fasciola* spp. in other provinces in South Africa as well as the parasite-intermerdiate host snail interactions.

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APPENDIX A: INFORMATION ON APPENDICES

Table 4: Identity and locality of *Fasciola spp.* used in this study and including Genbank sequences.

Sample	Locality	Identity
J1	KwaZulu-Natal (KZN)	f hepatica
J2		f hepatica
J3		f hepatica
J4		f hepatica
J5		f hepatica
J6		f hepatica
J7		f hepatica
J8		f hepatica
J9		f hepatica
J10		f hepatica
J11		f hepatica
J12		f hepatica
J13		f hepatica
J14		f hepatica
J15		f hepatica
J16		f hepatica
J17		f hepatica
J18		f hepatica
J19		f hepatica
J20		f hepatica
J21		f hepatica
J61	Belfast	f hepatica
J62		f hepatica
J63		f hepatica
J64		f hepatica
J65		f hepatica
J66		f hepatica
J67		f hepatica
J68		f gigantica
J69		f gigantica
J70		f gigantica
J71		f hepatica
J72		f hepatica
J73		f hepatica
J74		f gigantica
J75		f hepatica

J76		f hepatica
J77		f hepatica
J96	**Mpumalanga Highveld**	f hepatica
J97		f hepatica
J99		f hepatica
J100		f hepatica
J101		f hepatica
J102		f hepatica
J103		f hepatica
J104		f hepatica
J105		f hepatica
J106		f hepatica
J107		f hepatica
J108		f hepatica
J109		f hepatica
J110		f hepatica
J111		f hepatica
J112		f hepatica
J113		f hepatica
KT182290.1	**Zimbabwe (Zim)**	f gigantica
KT182292.1		f gigantica
KT182295.1		f gigantica
AB510491.1	**Egypt**	f hepatica
KT182304.1	**PMB (KZN)**	f hepatica
GU112458.1	**Niger**	f gigantica
GU112459.1		f gigantica
GU112470.1		f hepatica
GU112469.1		f hepatica
GU112472.1	**China**	f gigantica
GU112473.1		f gigantica
GU112484.1		f gigantica
GU112485.1		f gigantica
GU112487.1		Intermediate
GU112488.1		Intermediate
GU112489.1		Intermediate
GU112490.1		Intermediate
AF215860.1	**Outgroup**	Schistosoma_japonicum